Title

Regional heterogeneities of oligodendrocytes determine biased distribution pattern of Ranvier nodes along single axons in sound localization circuit

Ryo Egawa[1],
Kota Hiraga[1],
Ryosuke Matsui[2],
Dai Watanabe[2],
Hiroshi Kuba[1,*]

[1]Department of Cell Physiology, Graduate School of Medicine, Nagoya University, Nagoya 466-8550, Japan
[2]Department of Biological Sciences, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.
***Correspondence**: kuba@med.nagoya-u.ac.jp

Number of pages: 37
Number of Figures and Tables: 7 figures and 3 videos
Number of words in Abstract: 236, Text: 4683

Key words (Max 6):
oligodendrocyte heterogeneity
oligodendrogenesis
internodal length
3D morphometry
interaural time difference
brainstem auditory circuit

Abstract

Distribution of Ranvier nodes along myelinated axons is a critical determinant of conduction velocity, significantly influencing spike arrival timing and hence neural circuit function. The patterns of nodal distribution are not necessarily uniform but vary across brain regions and even along individual axons. Although various factors could contribute to these patterns, the complexity arising from multicellular interactions between oligodendrocytes and axons has left the underlying regulatory mechanisms unclear. In this study, we identified the factors contributing to the emergence of a biased nodal distribution pattern along single axons using the chick brainstem auditory circuit as a model, in which the distance between adjacent nodes (internodal length) varies along the axons of avian cochlear nucleus neurons in a region-dependent manner, thereby enabling precise binaural integration for sound localization. 3D morphometry revealed that these axons were almost fully myelinated by oligodendrocytes exhibiting distinct morphologies and cell densities across regions after hearing onset. The structure of axons did not affect internodal length. Inhibiting vesicular release from the axons did not affect internodal length and oligodendrocyte morphology, either, but caused unmyelinated segments on the axons by suppressing oligodendrogenesis near the presynaptic terminals. These results suggest that the regional heterogeneity in the intrinsic properties of oligodendrocytes determines the biased nodal distribution pattern in the sound localization circuit, while activity-dependent signaling supports the pattern by ensuring adequate oligodendrocyte density. Our findings highlight the importance of oligodendrocyte heterogeneity in fine-tuning neural circuit function.

Introduction

Ranvier nodes are highly excitable domains distributed along myelinated axons and contribute to increase conduction velocity through saltatory conduction. The distance between adjacent nodes (internodal length) affects the speed of saltatory conduction, with longer internodes leading to faster conduction, and hence has a significant impact on neural circuit function by influencing the timing of input arrival at target neurons. The pattern of nodal distribution along axons is not necessarily uniform. Recent studies have shown that the internodal length varies not only among axonal types and brain regions (Chong et al., 2012), but also along individual axons (Tomassy et al., 2014; Ford et al., 2015; Bonetto et al., 2021). Despite its potential importance in neural information processing, the mechanisms regulating nodal distribution patterns are not well understood.

Various factors could contribute to the emergence of nodal distribution patterns along axons. In the central nervous system (CNS), each Ranvier node is formed through the following processes. Immature oligodendrocytes contact axons with their processes, initiating myelin formation that

extends along the axon (Snaidero et al., 2014). At the ends of the extending myelin, the paranodal domain, at which the myelin tip contacts the axon, acts as a diffusion barrier and facilitates the clustering of voltage-gated sodium channels (Nav) and the scaffolding protein ankyrinG (AnkG) to form a heminode. Subsequent restriction of the gap between two neighboring heminodes forms a mature Ranvier node (Vabnick et al., 1996; Susuki et al., 2013; Rasband and Peles, 2015; 2021). Thus, the formation of nodal distribution along axons is based on multicellular interactions among oligodendrocytes and axons and could be influenced by multiple factors associated with these cells; the factors related to oligodendrocytes include their morphological characteristics (e.g., the number and length of myelin sheaths) and cell density relative to axons (Chong et al., 2012), while those related to axons include their branching, diameter (Bechler et al., 2015), and neural activity (Fields, 2015; Bechler et al., 2018; Bonetto et al., 2021; Osanai et al., 2022). In addition, the contributions of these factors would vary significantly due to the diversity of both cell types and developmental stages, which obscures our understanding of the mechanisms of determining the nodal distribution patterns.

To understand the regulatory mechanisms of nodal distribution, the chicken brainstem auditory circuit is an excellent model, because the nodal distribution is biased in a region-dependent manner along the same projection axons, minimizing the effects of neuronal diversity. In addition, this regional difference in internodal length is also important in the computation of neural circuits for sound localization. Sound localization is the ability to identify the direction of sound source and involves detecting the time difference in sound arrivals between the ears (interaural time difference, ITD) in the order of microseconds. The ITD detection is mediated by an array of coincidence detector neurons in the nucleus laminaris (NL) that receive excitatory synaptic inputs from both sides of the nucleus magnocellularis (NM), a homologue of mammalian anteroventral cochlear nucleus (Figure 1A) (Carr and Konishi, 1990; Hyson 2005). In this circuit, the distribution of nodes along the axon of NM neurons differs among regions; the internodal length is longer at the tract region across the midline than at other regions including the NL (Figure 1B) (Seidl et al., 2010). The long internodes reduce the conduction time from the contralateral side and compensate for the difference in axonal pathlengths between the two sides (Seidl et al., 2014), while the short internodes expand the dynamic range for detectable ITD within the NL. Therefore, this model will be an important stepping stone for elucidating the regulatory mechanisms of nodal distribution and their impact on neural circuit function.

In this study, we tested the potential factors contributing to the regional differences in the nodal distribution along NM axons using high-resolution 3D morphometry of the optically-cleared brainstem auditory circuit. The results showed that NM axons are almost fully myelinated by oligodendrocytes with distinct morphological features after hearing onset. This morphological difference was not due to axonal structure. Inhibition of vesicular release from NM axons did not affect the internodal length and oligodendrocyte morphology but caused unmyelinated segments on NM axons via a suppression of oligodendrogenesis at the NL region. These results identified that the main

factor contributing to the biased nodal distribution pattern in the ITD circuit is the regional heterogeneity in the intrinsic properties of oligodendrocytes. Activity-dependent signaling also contributed to this circuit by ensuring the density of oligodendrocytes through enhanced oligodendrogenesis. Our findings provide new insights into the mechanism of regulating nodal distribution along single axons and also into the significance of oligodendrocyte heterogeneity in neural circuit function.

Results

Ranvier nodes developed in a similar time course along NM axons after hearing onset

NM neurons in chick embryos form axonal projections to the NL at embryonic day 10 (E10), begin to receive auditory input from around E12, and are functionally mature by the time around hatch (Akter et al., 2018; 2020) (Figure 1C). To clarify how node formation progresses along development and whether there are regional differences in the timing of node formation, we examined the development of nodes at the tract and NL regions by double staining with AnkG and pan Nav antibodies or AnkG and Caspr antibodies, a marker for the paranodal domain (Figure 1D–F).

At E12, we did not find these molecules at each region. At E15, Nav-positive clusters appeared and they were localized specifically at one end of the fibrous AnkG signals. The remaining Nav-negative part of AnkG signals would correspond to the paranodal domain, as myelin sheath at the domain shows transient expression of AnkG during the immature period of CNS (Chang et al., 2014), and these structures were defined as 'heminode'. At E18, Caspr signals became detectable at the paranodal domain of heminode. In addition, some Caspr signals appeared on both sides of the fibrous AnkG signals, sandwiching the Nav cluster. These structures would be formed through the fusion of adjacent heminodes and were defined as 'immature node'. At E21, many nodes showed mature patterns, with complete colocalization of Nav and AnkG at a short nodal domain, which was flanked by Caspr-positive paranodal domains ('mature node'). Triple staining of these molecules confirmed the three stages of nodal development along the axon of NM neurons. Quantification of their abundance revealed that their maturation progressed gradually after hearing onset and was almost completed around hatch. 90 % of the node types were heminodes at E15. This decreased to about 50% by E18 and to about 10% by E21 (Figure 1G, H), suggesting that NM axons are almost fully myelinated by E21. Although the increase in the proportion of immature nodes was slightly preceded at the tract region, the overall tendency did not differ greatly between the regions. These data indicate that the formation of Ranvier nodes progress in a similar time course along the axon.

Development of myelin sheath along NM axons

Formation of nodes requires maturation of myelin sheaths. To clarify their relationship on NM axons, we also examined the development of myelin with an antibody against myelin associated glycoprotein (MAG) during the period of node formation.

The immunosignals for MAG appeared around the midline of the brainstem at E12 and extended along the axons of NM neurons at E15, becoming more intense at later ages for both tract and NL regions (Figure 2A, B). These are consistent with the time course of node formation. Importantly, many oligodendrocytes were situated along the axon, as identified by immunopositivity Olig2, a marker in the nucleus of oligodendrocyte lineage cells (Figure 2C). In addition, the MAG signals overlapped with the heminodes at E15. (Figure 2D), indicating that myelin sheaths already exist around the paranodal domains at the very early stage of node formation, in agreement with the idea that the early nodes can shift their position dynamically with myelin maturation (Figure 2E). These raised the questions as to when the biased nodal distribution pattern emerges during development and whether and how oligodendrocytes contribute to the process.

Internodal length was determined roughly during node formation

To answer these questions, we first evaluated the internodal length between mature/immature nodes at E18 and P9 at the tract and NL regions. These two time points correspond to the periods during and after node formation, respectively. We labeled axons with a tracer, rhodamine dextran, visualized nodes with the Caspr antibody, and measured the internodal length at each region (Figure 2F).

The internodal length was about 3 times longer in the tract region compared with the NL region at P9 (tract: 211.1±9.6 μm; NL: 63.9±3.0 μm) (Figure 2G), as observed in posthatch animals (Seidl et al., 2010). The differences in internodal length in these two regions of axons was already evident at E18, although the internodal length was shorter by 20% at E18 in both tract and NL regions (tract: 171.0±7.0 μm; NL: 53.4±3.8 μm). The slightly shorter internodal length at E18 would reflect an elongation of internode during development in accordance with maturation of the brain size. Thus, the nodal distribution pattern was already determined at the early period of node formation, implying that reorganization of internodes would not contribute to the biased internodal length.

Morphology of oligodendrocytes differed between tract and NL regions

We next explored the contributions of oligodendrocytes to the regional difference in internodal length by evaluating the morphology of individual oligodendrocytes at E21. We sparsely labeled mature oligodendrocytes by expressing tdTomato with a palmitoylation signal (paltdTomato) under MBP promoter using *in ovo* electroporation and the iOn switch (Kumamoto et al., 2020) (Figure 3A, B), while labeling bilateral NM axons with GFP by transfection of avian adeno-associated virus (A3V) (Figure 3C). The 3D morphology of oligodendrocytes was then observed in 200 μm-thick brainstem slices optically cleared with SlowFade Glass mountant (Figure 3D) (Video 1, 2). We focused our

analyses on oligodendrocytes ensheathing the main trunk of NM axons.

As presumed, length of myelin sheaths differed significantly between the tract and NL regions (tract: 174.1±5.0 μm; NL: 59.7±2.4 μm) (Figure 3E), which was consistent with the difference in internodal length between the regions (Figure 2B). Importantly, the diameter of myelin sheath including the axon did not differ between the regions and did not show any correlation with the myelin length (tract: 1.69±0.05 μm; NL: 1.64±0.07 μm) (Figure 3F, G). In each oligodendrocyte, the average myelin length was shorter by 3 times (tract: 181.1±7.3 μm NL: 63.6±4.7 μm), the number of myelin sheaths was larger by 2 times (tract: 3.0±0.2; NL: 6.6±0.6), and the total myelin length was shorter by 1.3 times (tract: 527.9±27.2 μm; NL: 394.6±29.7 μm) at the NL region compared to the tract region (Figure 3H–J). In addition, the cell body size was 30% smaller in cross-sectional area at the NL region (tract: 78.2±2.7 μm^2; NL: 52.5±1.9 μm^2) (Figure 3K). Thus, oligodendrocyte morphology was heterogeneous, with distinct differences in several aspects between the tract and NL regions (Figure 3L, M), even though they were myelinating the same axons.

Axon structure did not affect the regional difference in nodal distribution

The distinct oligodendrocyte morphologies imply differences in their intrinsic properties (e.g., the ability to produce and extend myelin sheaths), but the involvement of other external constraints cannot yet be ruled out. The structure of axons, such as branch points and diameter, is one of these external constraints, and can affect myelin extension (Bechler et al., 2015). We therefore investigated the relationship between the structural features of NM axons and the nodal distribution at E21. To accomplish this, we labeled the axon of NM neurons by expressing GFP with a palmitoylation signal (palGFP) using *in ovo* electroporation and examined the nodal distribution three-dimensionally along the axon in 200 μm-thick brainstem slices optically cleared after staining with a Caspr antibody (Figure 4A–D) (Video 3).

The results showed that on the main trunk of the axons, most of the Caspr signals showed mature/immature node patterns, confirming that the axons were almost fully myelinated. These axons formed multiple collateral branches innervating the ventral dendritic layer at the NL region, and triads of paranodal domains were frequently observed at the branch points. The internodal length (73.1±2.1 μm) being about 40% shorter compared to the branch point interval (122.5±6.7 μm) at the NL region (Figure 4E, F). The number of internodes between adjacent branch points averaged 1.6 and was correlated with the length of branch point interval (‘0’: 27.5±3.9 μm; ‘1’: 79.3±5.1 μm; ‘2’: 156.3±7.1 μm; ‘3≦’: 232.0±10.0 μm) (Figure 4H, I). In areas with very short branch point intervals (bottom 10%: <40 μm), the axon lacked Caspr signals frequently (61.5%), implying that these areas are less likely to be myelinated (internode=0 μm) (Figure 4G). On the other hand, even in areas with long branch point intervals (top 10%: >220 μm), the internodal length did not increase and it was 2 times shorter than that at the tract region (<220 μm: 71.4±2.4 μm; >220 μm: 79.9±3.9 μm; tract: 164.3±4.2 μm) (Figure 4J). These observations suggest that the branch point interval is not a critical determinant

of the short internodes at the NL region, although it can be a limiting factor in areas with the shorter branch point interval.

The diameter of axon main trunk did not differ between the tract and NL regions (tract: 1.40±0.03 μm; NL: 1.47±0.04 μm) (Figure 4K), corresponding to the diameter of myelin sheaths including axon (Figure 3F). This was consistent with the previous report showing that the axon diameter did not differ between the tract and the distal portion of ventral NL regions (Seidl et al., 2010), confirming that the diameter of NM axons is not a factor determining the regional differences in nodal distribution.

Oligodendrogenesis was augmented at NL region

Given that NM axons were fully myelinated, oligodendrocyte density could also be a constraint of individual oligodendrocyte morphology; if the density is high, it may limit the extension of myelin sheath due to mutual competition for territory on the axon. Therefore, we examined the density of oligodendrocyte lineage cells during development by labeling the cells with the Olig2 antibody and quantifying their density at the tract and NL regions between E9 and E21(Figure 5A top, B).

The results showed that the density of Olig2-positive cells increased with development, peaked at E15, and decreased slightly afterwards. Importantly, the extent of this increase was more robust and about 1.3-fold higher at the NL region than at the tract region. The differences in oligodendrocyte density could result from several factors, including the density of oligodendrocyte precursor cells (OPCs), which mature into oligodendrocytes through several steps of maturation, as well as the migration, proliferation, and differentiation of these cells. We measured the density of OPCs by labeling the cells with Nkx2.2 antibodies (Figure 5A bottom, C) (Xu et al., 2000). The density of Nkx2.2-positive cells increased sharply and reached a plateau by E12 at both regions, while the extent of this increase was also higher at the NL region. These cells were restricted to the ventral and dorsal fiber layers and almost absent in the somatic and dendritic layers of NL. These results suggest that the density of OPCs increases around the period of hearing onset to a larger extent at the NL region, contributing to the higher density of oligodendrocyte lineage cells at this region. We evaluated the level of proliferation of OPCs with BrdU labeling and found that the density of BrdU-positive cells was significantly higher at the NL region during E12–16 (Figure 5D, E). BrdU-positive cells were immunopositive for both Olig2 and Nkx2.2, confirming that they were OPCs (Figure 5F, G). These results suggest that the higher densities of OPCs and oligodendrocyte lineage cells at the NL region occur through a region-specific facilitation of oligodendrogenesis.

Inhibition of vesicular release did not affect internodal length but caused unmyelinated segments at NL region

Myelination could be modulated adaptively by neuronal activity (Fields, 2015; Bechler et al., 2018; Bonetto et al., 2021; Osanai et al., 2022). We here tested the possible contributions of activity-

dependent adaptive mechanisms to the nodal distribution at E21 by inhibiting vesicular release from NM axons through an introduction of enhanced tetanus neurotoxin light chains (eTeNT; Kinoshita et al., 2012) into bilateral NM neurons using A3V (Figure 6A–D).

The results showed that although Caspr-positive nodes were still observed in the axons expressing eTeNT, these axons showed an increase in the fraction of heminodes of about 30% causing unmyelinated segments at the NL region (A3V-GFP: 4.6±1.2%; A3V-eTeNT: 17.6±3.0%) (Figure 6D–G). Interestingly inhibiting vesicular release from axons by expressing eTeNT did not affect the internodal length at both tract and NL regions, and the length did not differ between those with and without heminodes (Figure 6H, I). These results indicate that the inhibition of vesicular release reduced the density of myelin sheaths with little effect on their length, thereby causing the unmyelinated segments at the NL region.

Inhibition of vesicular release did not affect oligodendrocyte morphology but suppressed oligodendrogenesis at NL region

The reduction of myelin sheath density by eTeNT could occur through a decrease in the number of myelin sheaths formed by each oligodendrocyte and/or through a decrease in the density of oligodendrocytes themselves. To test these possibilities, we sparsely labeled mature oligodendrocytes and evaluated the effects of eTeNT on their morphology at E21 (Figure 6J, K).

The results showed that eTeNT affected neither the number nor the length of myelin sheaths in each oligodendrocyte at the NL region, supporting the conclusion that the oligodendrocyte morphology is determined in region-specific but activity-independent manners along NM axons.

To determine if inhibiting vesicular release from axons alters proliferation of OPCs, we examined the density of Nkx2.2-positive OPCs and BrdU-positive proliferating cells at E15 in the A3V-eTeNT and control (A3V-GFP) conditions (Figure 6L, M). In the control group, the density of these cells became higher by 1.5 folds at the NL region compared to the tract region (see also Figure 5C, E). In the eTeNT group, on the other hand, this enhancement was suppressed, which abolished the difference in the density of these cells between the regions. These data indicate that vesicular release from NM axons promotes oligodendrogenesis specifically at the NL region without altering oligodendrocyte morphology, suggesting that adaptive oligodendrogenesis is important in covering the entire length of the axon with short myelin sheaths at the region (Figure 7A–C).

Discussion

This study identified several factors responsible for biased nodal distribution patterns along axons, including heterogeneity in oligodendrocyte morphology, regional differences in proliferation of OPCs, and activity-dependent signaling from axons through vesicular release.

Using the chick brainstem auditory circuit known for its characteristic nodal distribution as

a model, we found that NM axons were almost fully myelinated by oligodendrocytes with distinct morphologies between the two regions having markedly different internodal lengths. This regional heterogeneity reflected differences in the intrinsic property of oligodendrocytes in these two regions rather than the extrinsic constraints. Inhibiting vesicular release from NM axons caused unmyelinated segments by region-specific suppression of oligodendrogenesis without affecting internodal length or oligodendrocyte morphology, suggesting that primary role of neural activity is to ensure the oligodendrocyte density necessary for full myelination of axons.

Biased nodal distribution pattern reflects regional heterogeneity of oligodendrocyte

The main factor contributing to regional differences in internodal length along NM axons was regional heterogeneity in oligodendrocyte morphology. The morphological differences were observed not only in the length of myelin sheaths, but also in the number of myelin sheaths and the size of cell bodies (Figure 3). Considering the morphological classification of oligodendrocytes by Pío del Río-Hortega (1928), oligodendrocytes at the tract and NL regions could be classified into type III and type II, respectively. Such morphological heterogeneity could be related to the variation of gene profiles among oligodendrocytes (Butt et al., 1998; Marques et al., 2016; Osanai et al., 2022). Differences in the pericellular microenvironment and/or the origin of OPCs during development may contribute to the diverse gene profiles of oligodendrocytes (Crawford et al., 2016; Foerster et al., 2019, 2024; Boshans et al., 2020; Sherafat et al., 2021). Indeed, oligodendrocytes in the brainstem have two developmental origins, derived from the ventral and dorsal sides of the hindbrain (Vallstedt et al., 2005). Whether oligodendrocytes and OPCs at the tract and NL regions differ in their origins and/or gene profiles is an important issue to be examined in the future.

The regional differences in the morphology of oligodendrocytes would reflect their intrinsic properties at each region; the ability of myelin production is higher, while that of myelin extension is lower at the NL region than at the tract region. Supportively, the axonal factors, such as structure and vesicular release, did not affect the regional heterogeneity in oligodendrocyte morphology (Figure 4, 6). Oligodendrocyte density did not affect the morphology, either; the number and length of myelin sheaths did not increase even when unmyelinated segments appeared after inhibition of vesicular release. Thus, the morphology of oligodendrocyte and hence the length of myelin sheaths, are determined according to the intrinsic properties of oligodendrocytes at each region, which underlies the regional differentiation of nodal distribution along NM axons. Variations in the intrinsic properties of oligodendrocytes are also reported in other brain regions. Oligodendrocytes in white matter are morphologically distinct from those in gray matter, and their original features did not change even when OPCs were cross-transplanted (Viganò et al., 2013). In addition, OPCs collected from different brain regions show their original morphological features when cultured on artificial fibers (Bechler et al., 2015). In summary, our findings provide the perspective that oligodendrocytes with different intrinsic properties work together to fine-tune the neural circuit function involved in ITD detection.

Adaptive oligodendrogenesis ensures full myelination of axons at NL region

Neuronal activity can affect oligodendrocyte maturation and morphology, but the effects are not uniform (Osanai et al., 2022). Indeed, the effect of the inhibition of vesicular release from axons on myelination is known to depend on the type of neurons (Koudelka et al., 2016). In the axons of NM neurons, the activity-dependent signaling did not affect myelin formation in mature oligodendrocytes but facilitated proliferation and differentiation of OPCs. Given that the enhancement of oligodendrogenesis was confined to the NL region and that A3V transduction into NM neurons was highly efficient (about 90%, comparable to previous reports; Matsui et al., 2012), exocytosis from the presynaptic terminals of NM axons caused the effects by altering the perisynaptic microenvironment. Glutamate and several trophic factors such as BDNF, which are released from the terminals of NM axons, may act on the OPC (Du et al., 2003; Pease-Raissi & Chan, 2021; Fekete & Nishiyama, 2022) directly via spillover from the synapse and/or indirectly via activation of NL neurons or astrocytes. Supportively, mRNA of NR1, a major subunit of NMDA receptor, is strongly expressed in glial cells, presumably oligodendrocyte lineage cells, at the ventral and dorsal dendritic layers of NL after E10 (Tang and Carr, 2007). Future studies are needed to determine what ligands mediate the differentiation and proliferation of OPCs as the activity-dependent signaling and whether there are regional differences in the sensitivity of these OPCs to the ligands along the NM axon.

Adaptive oligodendrogenesis would support the function of brainstem auditory circuits by ensuring the full myelination of NM axons. Full myelination of the auditory pathway enables rapid membrane responses and high metabolic support during high-frequency firing, which are critical for precise and reliable processing of sound information (Kim et al., 2013; Moore et al., 2020). Moreover, the full myelination at the NL region, which is mediated by the adaptive enhancement of oligodendrogenesis, would be particularly important for ITD detection. The contralateral projections of NM neurons have a sequential branching structure called a “delay line” at the ventral area of NL in chickens. This delay line produces an output delay of up to 180 μs for contralateral NL neurons along the medial to lateral axis (Overholt et al., 1992). This delay covers the physiological ITD range for the chicken, allowing the ITDs to be encoded in a “place map” within the NL (Jeffress, 1948; Köppl & Carr, 2008). This should provide the neural basis for the ability of excellent sound localization in chickens, which can discriminate ITDs of about 20 μs (8.9° minimum audible angle) (Krumm et al., 2022). Reliable myelination of the delay line ensures constant conduction velocity and output delay and hence is critical for precise ITD calculation. Thus, the adaptive oligodendrogenesis at the NL region would be a mechanism that secures the accuracy of ITD calculation in the avian auditory circuit. Interestingly, ITD representation within the NL exhibits plasticity around the period of hearing onset in the barn owl (Carr et al., 2024), which coincides with the period of adaptive oligodendrogenesis, speculating its contribution to the plasticity.

Commonalities and differences with the sound localization circuit in mammals

Regional regulation of conduction velocity has also been reported in mammals. In a brainstem auditory circuit of gerbils, which is involved in the processing of ITDs, several regional differentiations have been observed in the nodal distribution along the axon. In the axon of spherical bushy cells, a mammalian homologue of NM neurons, the internodal length is longer for contralateral side than for ipsilateral side, presumably compensating for the different axonal pathlength between the two sides (Seidl & Rubel, 2016). On the other hand, in the axon of globular bushy cells that project to medial nucleus of trapezoid body, the internodal length is longer for those innervating more medial location (i.e. more distal location), ensuring simultaneous inputs within the nucleus (Ford et al., 2015). These regional differentiations of nodal distribution would be acquired independently in mammals and birds through evolutionary convergence (Lipovsek & Wingate, 2018), emphasizing their importance in securing ITD computation in the microsecond order. Notably, in globular bushy cells, neural activity did not alter the nodal distribution, but instead regulated the conduction velocity by adaptively changing the axon diameter (Nabel et al., 2024), suggesting that the mechanism of action of activity-dependent signaling is different among neurons and species. It will be interesting to determine to what extent the intrinsic properties of oligodendrocytes and other extrinsic constraints contribute to the nodal distribution in mammalian ITD circuits. Such comparisons will help to understand the fundamental logic and mechanisms of determining nodal distribution patterns along the axons.

Conclusion

This study identified the factors mediating the regional differentiation of nodal distribution along single axons in the auditory pathway of chicken; the differentiation of nodal distribution reflects regional heterogeneity in oligodendrocyte morphology, and the activity-dependent signaling from axons via vesicular release contributes to the full myelination of axons through region-specific enhancement of oligodendrogenesis. The results in this model system provide evidence that oligodendrocyte heterogeneity, which is widely observed in the brain, can contributes to achieving optimal timing of signals and optimal function of local circuits in the nervous system.

Materials and methods

Animals

Chickens (*Gallus domesticus*) of either sex between embryonic day 9 (E9) and posthatch day 9 (P9) were used for experiments. The care of experimental animals was in accordance with the regulations on animal experiments at Nagoya University and the experiments were approved by the institutional committee. Fertilized eggs were incubated in a humidified incubator at 37.5°C to desired stage. The developmental stage of embryos was determined according to the Hamburger and Hamilton (1951) series. Chicks were deeply anesthetized with Isoflurane (FUJIFILM Wako, Japan), and embryos were anesthetized by cooling eggs in ice-cold water. Brainstem tissues containing middle-one third of NM were mostly used for experiments (Kuba et al., 2005).

Immunohistochemistry

Mouse pan Nav antibody (5 µg/ml, Sigma), rabbit AnkG antibody (5 µg/ml, a gift from Gisèle Alcaraz, Bouzidi et al., 2002), mouse Caspr antibody (2 µg/ml, NeuroMab), mouse MAG antibody (2 µg/ml, Merck), rabbit Olig2 antibody (2 µg/ml, a gift from Hirohide Takebayashi, Takebayashi et al., 2000), mouse Nkx2.2 antibody (2 µg/ml, DSHB), rat BrdU antibody (10 µg/ml, Abcam), rabbit GFP antibody (2 µg/ml, MBL), rat GFP antibody (2 µg/ml, Santa Cruz) , rabbit RFP antibody (2 µg/ml, Rockland) were used for immunohistochemistry. Chicks were perfused transcardially with a periodate-lysine-paraformaldehyde fixative (ml/g body weight): 1% (w/v) paraformaldehyde, 2.7% (w/v) lysine HCl, 0.21% (w/v) $NaIO_4$, and 0.1% (w/v) Na_2HPO_4. The brainstem was postfixed for 1.5 hours at 4 ˚C. After cryoprotection with 30% (w/w) sucrose in PBS, coronal sections (20–30 µm) were obtained. The sections were incubated overnight with the primary antibodies, then with Alexa-conjugated secondary antibodies (10 µg/ml, Molecular Probes) for 2 hours and were observed under a confocal laser-scanning microscope (FV1000, Olympus). For each image, 6-9 confocal planes were Z-stacked with a step of 0.8 µm. Images were analyzed in Fiji (Schindelin et al., 2012).

Anterograde labeling of NM axons was made by injecting dextran (MW 3000) conjugated with TMR (Life Technologies, 10–40% in 0.1 M phosphate buffer adjusted to pH 2.0 with HCl) into the midline tract region through a patch pipette and incubating the brainstem in the HG-ACSF for 30 min at 38 ˚C (Lawrence and Trussell, 2000; Wirth et al., 2008).

Internodal length was measured as follows. Images were captured with a ×60, 1.35-NA objective (Olympus), and 6-10 confocal planes were Z-stacked with maximum projection. Internodal length was defined as a distance between adjacent nodes, which determined by identifying a pair of Caspr signals. Number of oligodendrocytes and their precursors was measured as follows. Images were captured with a ×20, 0.75-NA objective (Olympus), and a single confocal plane was used for the measurement. The number was measured automatically by setting a threshold in areas over 14000 μm^2 at each region.

BrdU labeling

BudU (10 mg/ml in PBS, Nacalai, Japan) was injected subcutaneously into embryos (0.1 mg/g body weight) one hour before fixation, and sections were prepared as described above. The sections were mounted on a cover slide, treated with 2M HCl for 40 min at 60 ˚C, and used for immunohistochemistry.

Plasmids

Following plasmids were used for *in ovo* electroporation: iOn-MBP∞paltdTomato, pCAG-hyPBase, pCAFNF-palGFP-WPRE and Atoh1-Flpo (a gift from Marcela Lipovsek, Lipovsek & Wingate, 2018). All plasmids were constructed by inserting the following sequences into the plasmid backbones of iOn-CAG∞MCS (Addgene #154013, Kumamoto et al., 2020), pCAG-EGFP-WPRE and pCAG-floxedSTOP-tdTomato-WPRE (Egawa and Yawo, 2019) using In-Fusion® Snap Assembly Master Mix (Takara) or NEBuilder® HiFi DNA Assembly Master Mix (NEB); 1.9 kb sequence of mouse MBP promoter (a gift from Yasuyuki Osanai), hyPBase (pCMV-hyPBase, a gift from Wellcome Trust Sanger Institute; Yusa et al., 2011), CAFNF sequence (pCAFNF-GFP, addgene #13772; Matsuda et al., 2007), palGFP (pAAV2 SynTetOff-palGFP, a gift from Hiroyuki Hioki; Sohn et al., 2017). pA3V-RSV-EGFP and pA3V-RSV-EGFP.eTeNT were used for A3V production. pA3V-RSV-EGFP.eTeNT was constructed by inserting eTeNT sequence (HiRet-TRE-EGFP.eTeNT; Kinoshita et al., 2012) into the plasmid backbones of pA3V-RSV-EGFP (Matsui et al., 2012). All constructs were verified by Sanger sequencing.

In ovo electroporation

Plasmids were introduced into chick embryos in the same manner as previously reported (Jahan et al., 2023). Briefly, plasmid cocktail (0.4–0.5 μg/μl of each) was injected into the neural tube of chick embryos at E2 (HH Stage 10–12; Hamburger and Hamilton, 1951) and introduced into the right side of the hindbrain (rhombomere 3–8). The electrical pulses were applied using a pair of electrodes (CUY613P1, NEPAGENE) placed in parallel at 2 mm apart. The settings of electroporator (NEPA21, NEPAGENE) were as follows; poring pulse: 15 V, 30 ms width, 50 ms interval, 3 pulses, 10% decay and transfer pulse: 5 V, 50 ms width, 50 ms interval, 5 pulses, 40% decay.

A3V transfection

A3V-GFP and A3V-eTeNT were prepared as previously reported (Matsui et al., 2012). Their yield was approximately 10^{13} genome copies (GC)/mL. Viral solution containing 0.05% Fast Green (Nakarai tesque) was injected into the lumen of neural tube near the ear vesicle at E2–3 (60–70 hours of incubation, HH stage 15–16), at which A3V infects NM neurons with high efficiency (Matsui et al., 2012).

Immunostaining and 3D-imaging of 200 μm-thick brainstem slices

Chick embryos were perfused transcardially at E21 with PBS containing 10 U/mL heparin followed by 4% (w/v) paraformaldehyde in PBS. The brainstem was postfixed overnight at 4 ˚C. After cryoprotection with 30% (w/w) sucrose in PBS, coronal sections (200 μm thickness) were obtained. The brainstem slices were delipidated and permeabilized with cold acetone for 3 min, washed in PBS containing 0.1% (w/v) NaBH4 for 5 min to remove autofluorescence, and stained with primary antibody for 3 days, followed by secondary antibody for 1 day. The stained slices were embedded with SlowFade Glass Soft-set Antifade Mountant (Thermo Fisher Scientific) using 200 μm-thick silicone rubber spacer and No.0 coverslip (CG00C2, ThorLab), thereby making them transparent.
3D images were captured with a ×60, 1.42-NA oil-immersion objective (UPLXAPO60XO, Olympus) or ×100, 1.5-NA oil-immersion objective (UPLAPO100XOHR , Olympus) under a spinning disk confocal laser-scanning microscope (SpinSR10, Olympus). For each image, approximately 600 confocal planes were Z-stacked with a step of 0.3 μm. Multiple 3D images were stitched together using Imaris Stitcher (Oxford Instruments).

3D morphometry

In the 3D images captured by SpinSR10, myelin length, internode length, and branch point interval were traced using SNT (Arshadi et al., 2021), a Fiji plugin. Oligodendrocytes presumed not myelinating NM axons (e.g., oligodendrocytes that are not present within NM axon bundles or whose myelin sheaths barely along the axon bundle) were excluded from the analysis. For myelin and axon diameters, the length between intensity peaks of membrane-localized fluorescent proteins on a line orthogonal to the axon was measured. For nodal distribution along a single axon, three different axonal segments were defined according to the types of nodes at the ends; “internode w/o heminode” was a segment between mature/immature nodes, “internode w/ heminode” was a segment either between a mature/immature node and a heminode, or between heminodes flanking unmyelinated segments when the axon was separated by multiple heminodes, and “unmyelinated segment” was a non-overlapping segment between heminodes consisting of adjacent internodes. Quantified data were graphed using PlotsOfData (Postma et al., 2019) or Excel (Microsoft).

Statistics

Normality of data and equality of variance were evaluated by Shapiro-Wilk test and F-test, respectively. Statistical significance was determined by two-tailed Student’s *t*-test or Wilcoxon rank sum test for comparison between two groups with normal or non-normal distributions, respectively. ANOVA or Kruskal-Wallis test was used for comparison among more than two groups with normal or non-normal distributions, respectively, and *post hoc* Tukey test or Steel-Dwass test was used for pair-wise comparisons. Chi-squared test was used to compare the proportions between groups. The level of

statistical significance was set at 0.05. Values are presented as the mean ± standard error (n = number of cells).

Acknowledgments

Special thanks to R. Douglas Fields and Sian Lewis for their insightful feedback and suggestions on this manuscript. We acknowledge Division for Medical Research Engineering, Nagoya University Graduate School of Medicine for usage of SpinSR10, Imaris Stitcher and NanoDrop 2000. We thank Gisèle Alcaraz and Hirohide Takebayashi for kindly distributing AnkyrinG and Olig2 antibodies. We also thank Kazuhiro Nakamura and Chika Nishimura for cooperation in A3V production. Further thanks are extended to Connie Cepko, Jean Livet, Marcela Lipovsek, Hiroyuki Hioki, Yasuyuki Osanai and Wellcome Trust Sanger Institute for a gift of pCAFNF-GFP (Addgene #13772; Matsuda et al., 2007), iOn-CAG∞MCS (Addgene #154013; Kumamoto et al., 2020), Atoh1-Flpo (Lipovsek & Wingate, 2018), pAAV2 SynTetOff-palGFP, 1.9-kb MBP promoter, and pCMV-hyPBase (Yusa et al., 2011) respectively. This work was supported by Grants-in-aid from MEXT KAKENHI (19H04747, 21H02577, 22K19358 and 24H00584 to HK; 17K07039, 20K15915 and 23K05986 to RE) and the Takeda Science Foundation to HK.

Competing interests

The authors declare no competing financial interests.

Author contributions

RE, HK designed the work.

RE, HK performed histochemistry.

RE, KH traced internode length.

RE made plasmid vectors, performed electroporation and 3D analysis

RM, DW made the A3V vectors.

RE, HK wrote the paper.

All authors edited the manuscript, read and approved the final manuscript.

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Figures and figure legends

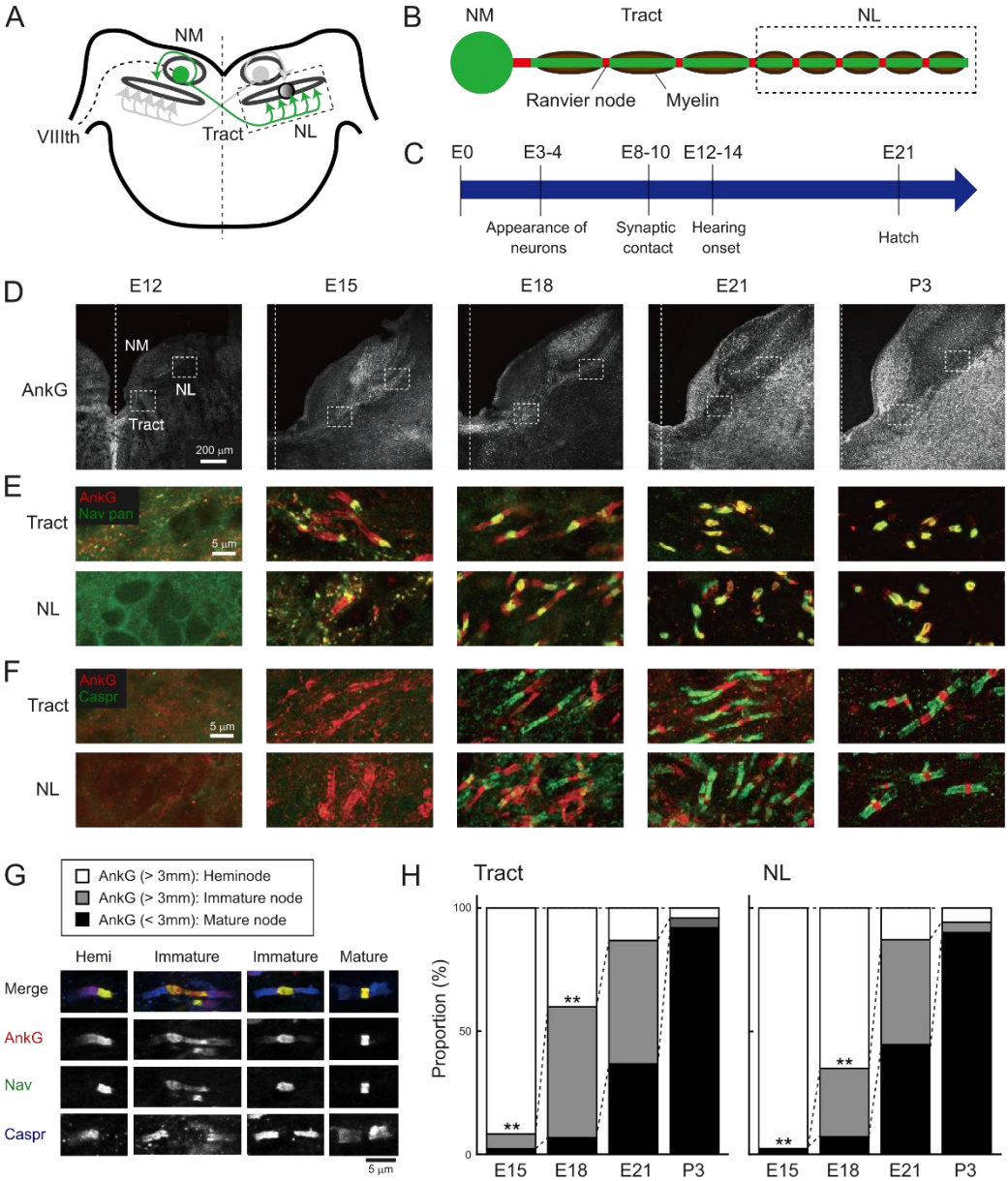

Figure 1. Development of Ranvier nodes progressed in a similar time course along NM axons after hearing onset.

(A) Axonal projection of NM neurons in the chicken brainstem. Main trunk of the axon projects to both sides of NL and forms "delay line" at contralateral NL. VIIIth, auditory nerve.

(B) Distribution of Ranvier nodes along the axon. Internodal length is long at midline tract region and becomes shorter at NL region in the axon.

(C) Development of NM neurons. NM neurons make synaptic contact with NL neurons by E10 and receive synaptic input from the auditory nerve by E12.

(D) Immunostaining of AnkG between E12 and P3. Dashed line indicates midline.

(E–F) Double immunostaining of AnkG (red) and panNav (green) for (E), and AnkG (red) and Caspr (green) for (F) at tract (upper) and NL (lower) regions from the same slices.

(G–H) Ranvier nodes matured on a similar time course across the region. Three types of Ranvier nodes immunostained with AnkG (red), panNav (green), and Caspr (blue) antibodies. (G), and their proportions between E15 and P3 (H). These types were determined according to the length of AnkG signals; the signals longer than 3 μm were defined as heminode or immature node according to the number of Nav-negative paranodal domains, and those shorter than 3 μm were defined as mature node. Note that some immature nodes had long nodal domains that exceeded 5 μm. This may correspond to a gap between the two heminodes. Proportions of node types at each developmental stage were compared between regions by chi-square test: $^{**}p < 0.01$. Tract: n=183, 344, 267, 226 at E15, 18, 21 and P3, respectively. NL: n=177, 267, 371, 412 at E15, 18, 21 and P3, respectively.

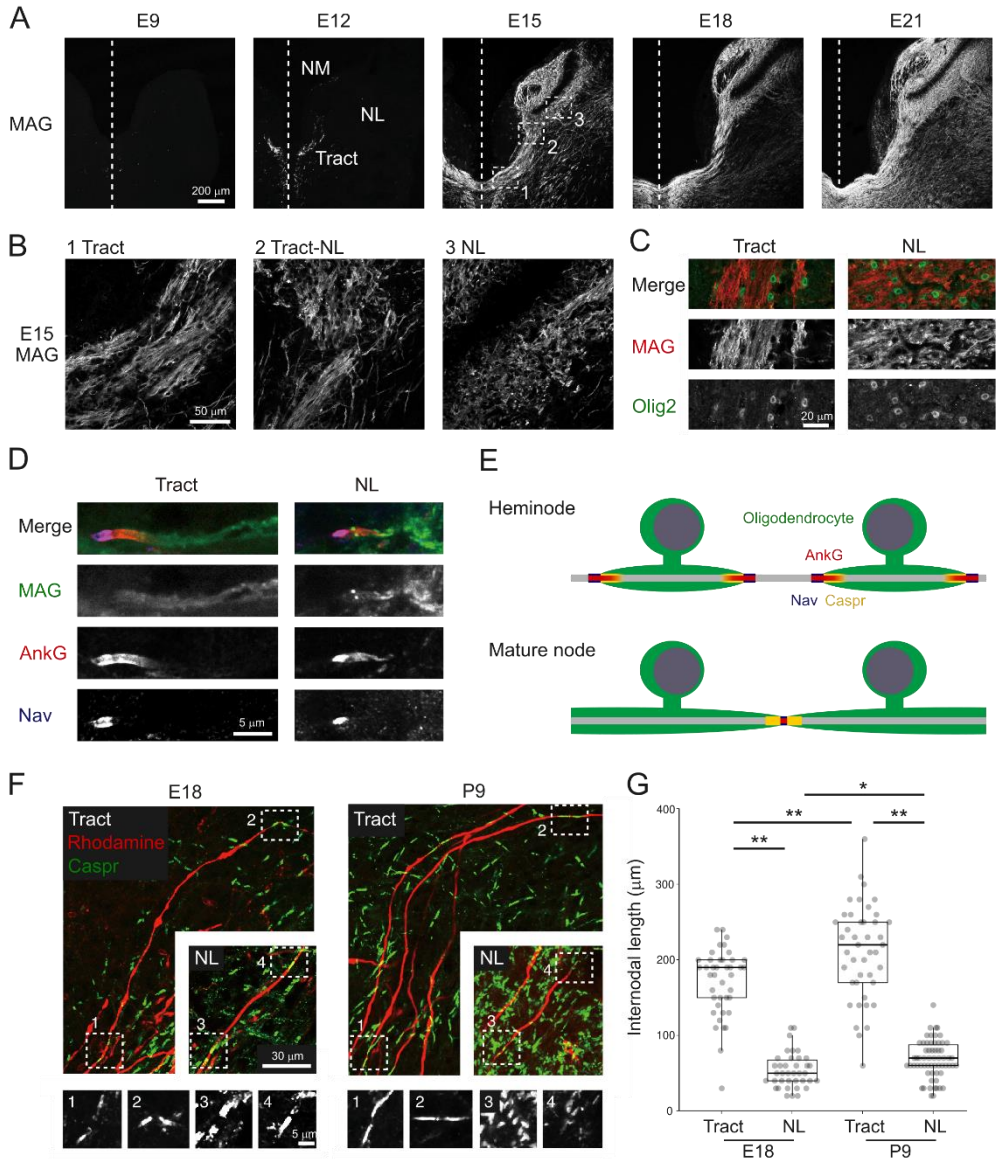

Figure 2. Regional difference in internodal length was evident during development.

(A–B) Immunostaining of MAG between E9 and E21 (A). Boxes with numbers at E15 are magnified (B).

(C) Double immunostaining of MAG (red) and Olig2 (green), a marker of oligodendrocyte lineage cells, at E15.

(D) Localization of MAG (green), AnkG (red), and Nav (blue) on heminode at E15.

(E) Ranvier node would be formed by restricting a gap between adjacent two heminodes during development. Note that paranodal domains, flanking the Nav-positive nodal domain, accompany fibrous AnkG clustering in heminode but not in mature node.

(F–G) Regional difference in internodal length appeared during the period of node formation. The

axons were labeled with rhodamine dextran (red) and stained with Caspr antibody (green) at E18 and P9 (F). Internodal length was measured as a distance between adjacent mature/immature nodes (G). Boxes with numbers correspond to high-magnification images of each node. Kruskal-Wallis test and post hoc Steel-Dwass test: *$p < 0.05$, **$p < 0.01$. E18: n=41 at tract, n=38 at NL. P9: n=43 at tract, n=62 at NL.

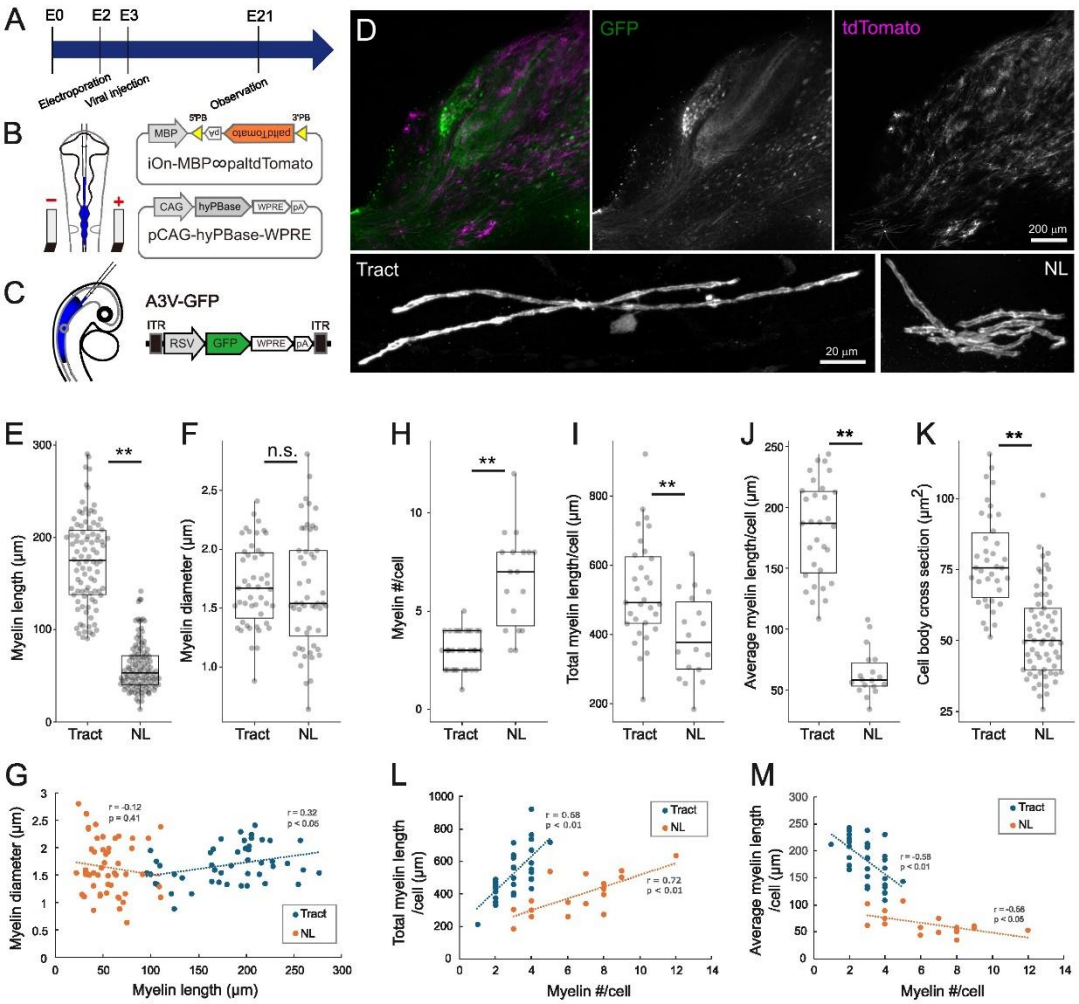

Figure 3. 3D morphometry of oligodendrocytes showed distinct differences between tract and NL regions.

(A) Timeline of experiments. *In ovo* electroporation and A3V transfection were performed at E2 (HH stage 11–12) and E2–3 (HH stage 15–16), respectively, and brainstem was observed at E21.

(B) Two types of plasmid vectors were introduced to visualize mature oligodendrocytes using *in ovo* electroporation. iOn-MBP ∞ paltdTomato expresses tdTomato with a palmitoylation signal (paltdTomato) under the control of the MBP promoter after the genomic integration. pCAG-hyPBase integrates the above plasmid into the genome by expressing hyperactive piggyBac transposase.

(C) A3V-GFP was injected into neural tube to visualize the axon of NM neurons.

(D) GFP and paltdTomato expressions in a 200-μm-thick slice (upper) and magnified images of single oligodendrocytes at tract and NL regions (lower). NM neurons and their axons were densely labeled with GFP (green), while mature oligodendrocytes were sparsely labeled with paltdTomato (magenta).

(E–G) Myelin morphometry showed concordance with internodal length and their uncorrelation with myelin diameter. Length (E), diameter (F) of myelin sheaths, and their relationship (G). These

parameters did not correlate with each other at both regions. E: n=94 at tract, n=119 at NL. F–G: n=47 at tract, n=52 at NL.

(H–M) Oligodendrocyte morphometry highlighted their regional heterogeneity. Number (H), total length (I), average length of myelin sheaths (J) per oligodendrocyte, and cross-sectional area of cell body **(**K**)**. Total (L) and average (M) lengths against number of myelins. Total and average myelin lengths showed increasing and decreasing tendencies, respectively, with an increase of myelin processes. H–J: n=31 cells at tract, n=18 cells at NL. K: n=38 cells at tract, n=65 cells at NL.

Two-tailed T-test: *$p < 0.05$, **$p < 0.01$, n.s., not significant.

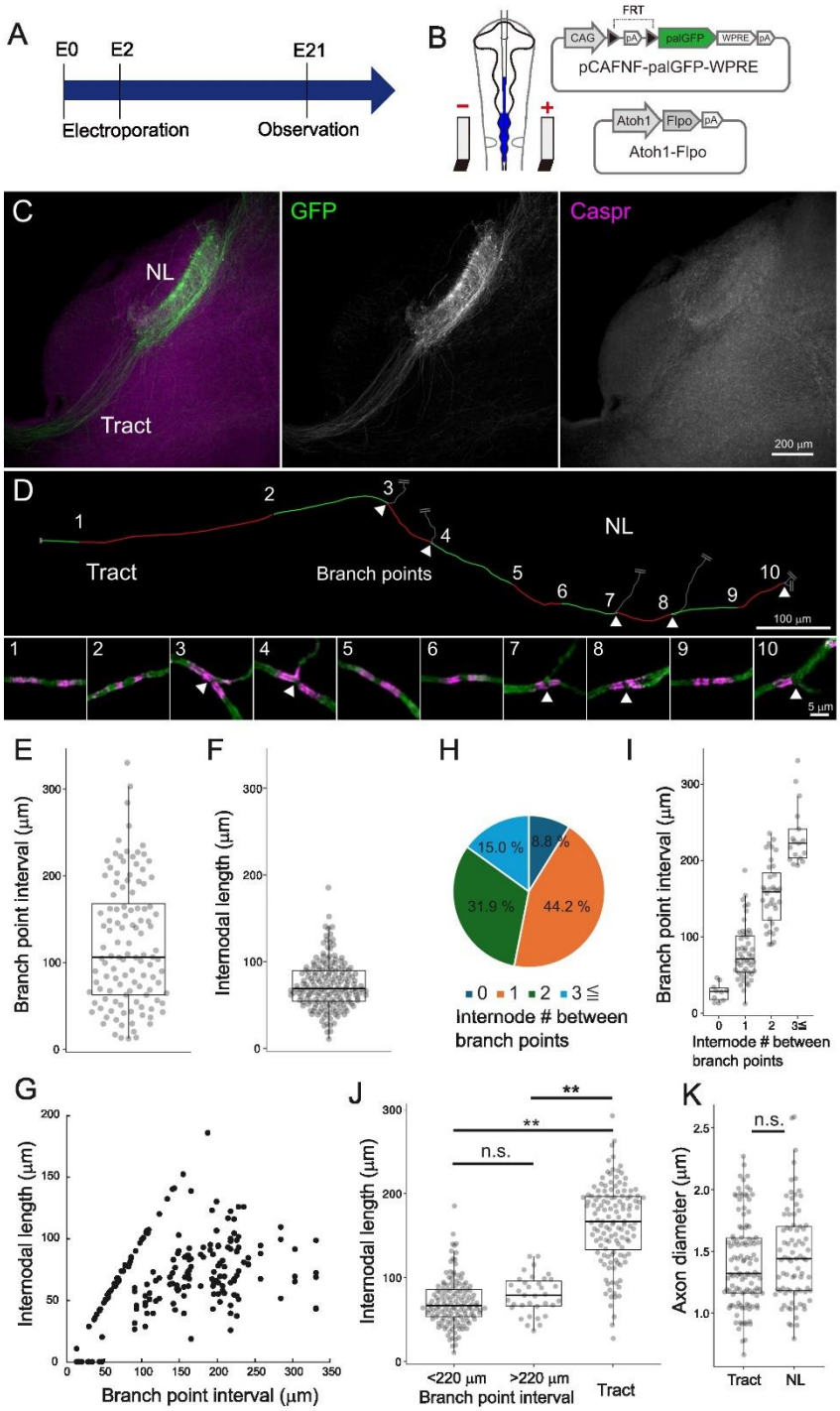

Figure 4. Axonal structure was not a major determinant of regional difference in nodal distribution.

(A) Timeline of experiments. *In ovo* electroporation was performed at E2 (HH Stage 11−12), and brainstem was observed at E21.

(B) Two types of plasmid vectors were introduced to visualize the axon of NM neurons using *in ovo*

electroporation. Atoh1-Flpo expresses Flpo under the control of Atoh1 promoter, which has selectivity for NM neurons. pCAFNF-palGFP-WPRE expresses GFP with a palmitoylation signal (palGFP) in a Flpo-dependent manner.

(C) Contralateral projections of NM neurons were sparsely labeled with palGFP (green), while visualizing paranodes with Caspr antibody (magenta) in a 200-μm-thick slice.

(D) Nodal distribution across tract and NL regions along a single NM axon. Arrowheads indicate branch points of collaterals. Each number corresponds to high-magnification images of node (lower panels). Each internode (between nodes) on the axon was labeled alternately with red and green lines, and their lengths were measured.

(E–G) Internodal length was clearly shorter than the branch point interval at the NL region. Branch point interval (E), internodal length (F) at NL region, and their relationship (G). Note that the internodal length was mostly below 100 μm at NL region even when the branch point interval was above 100 μm. E: n=113, F: n=178, G: n=188.

(H–I) Branch point interval correlated with the number of internodes contained therein. Number of internodes between adjacent branch points (H) and branch point interval against the number of internodes (I). “0”: n=10; “1”: n=50; “2”: n=36; “3≦”: n=17.

(J) Internodal length was not necessarily specified by branch point interval. Comparison of internodal length for branch point intervals above 220 μm (top 10% of measured values), below 220 μm at NL region, and at tract region. <220: n=143, >220: n=35, Tract: n=132.

(K) Diameter of main trunk of the axon at tract and NL regions was not different. Tract: n=114, NL: n=81.

One-way ANOVA and post hoc Tukey test (J) and two-tailed T-test (K): *$p < 0.05$, **$p < 0.01$, n.s., not significant.

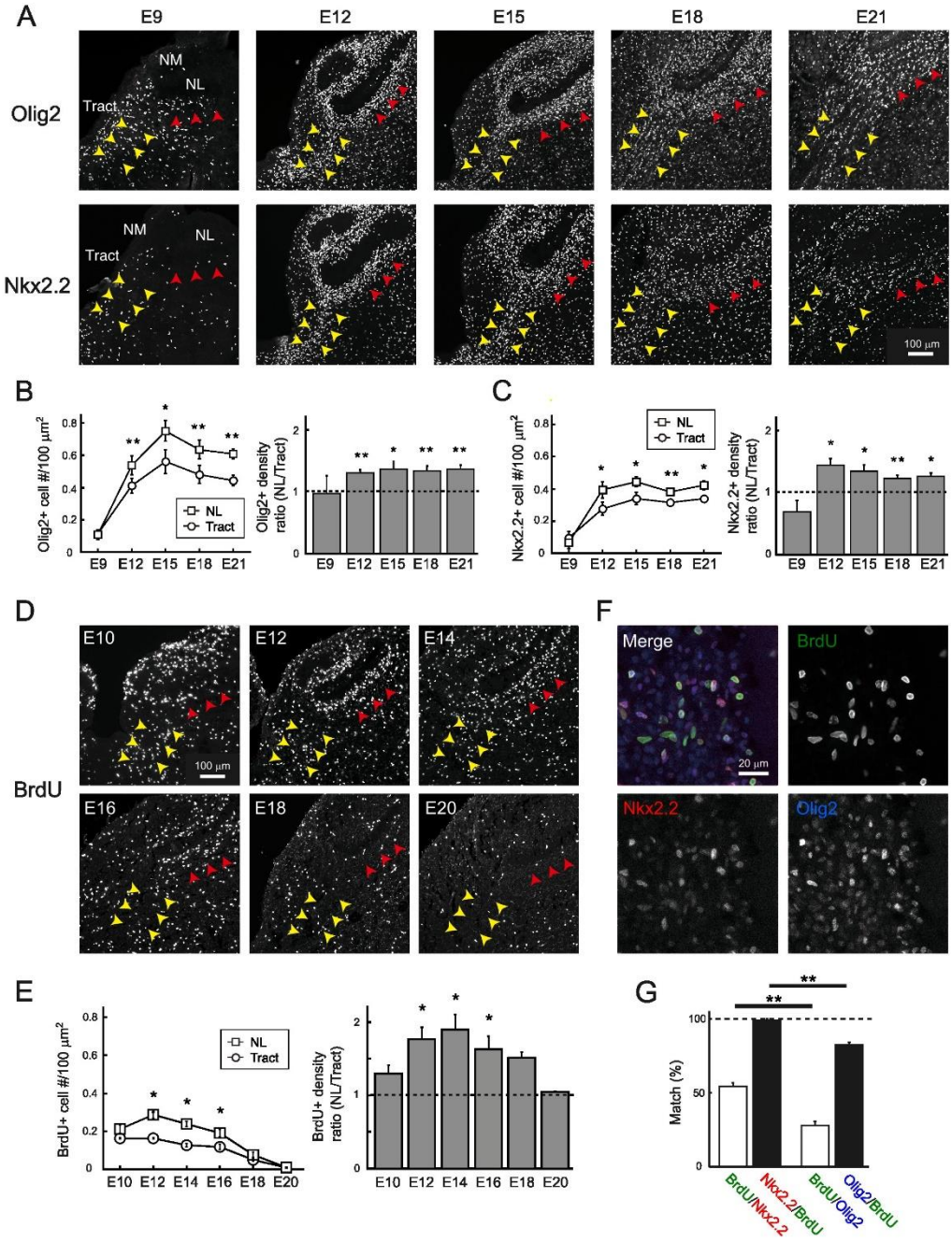

Figure 5. Region-specific facilitation of oligodendrogenesis led to higher oligodendrocyte density at NL region.

(A) Immunostainings of Olig2, a marker for oligodendrocyte lineage cells, and Nkx2.2, a marker for OPCs. Yellow and red arrowheads indicate tract and NL regions, respectively.

(B–C) Density of developing oligodendrocyte increased especially at the NL region. Developmental changes in the density of (B) Olig2-and (C) Nkx2.2-positive cells at each region and their ratio between the regions. N=3–6 chicks for each stage.

(D) Immunostainings of BrdU, a marker for proliferating cells. Yellow and red arrowheads indicate tract and NL regions, respectively.

(E) Cell proliferation was facilitated at the NL region. Developmental changes in the density of BrdU-positive cells at each region and their ratio between the regions. N=3 chicks for each stage.

(F) Colocalization of BrdU (green), Nkx2.2 (red), and Olig2 (blue) signals at E12.

(G) Proliferating cells were almost exclusively OPCs. Percentage of BrdU-positive cells among Nkx2.2- or Olig2-positive cells, and the percentage of Nkx2.2- or Olig2-positive cells among BrdU-positive cells at E12–14. N=4 chicks.

Two-tailed T-test: *$p < 0.05$, **$p < 0.01$.

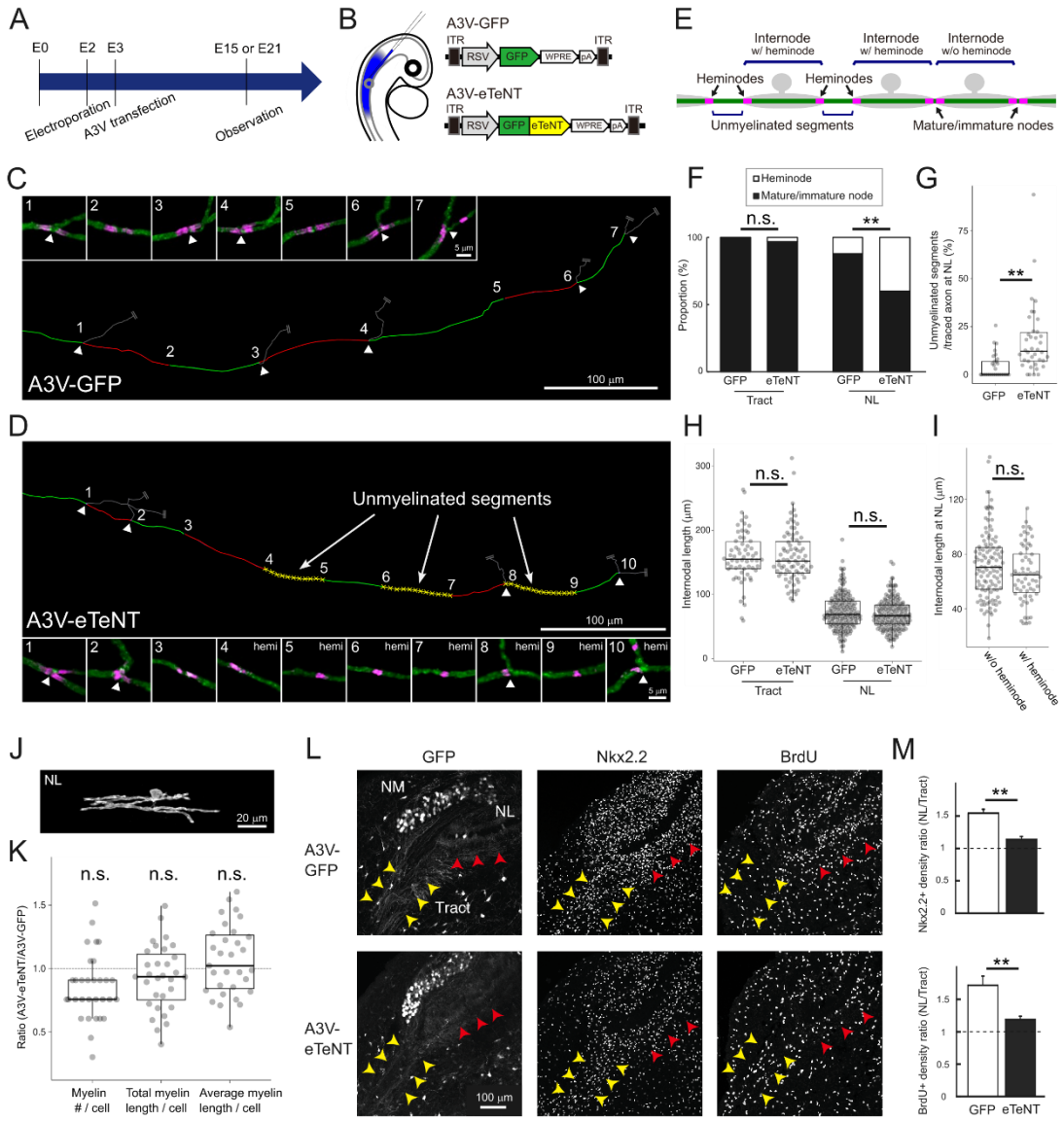

Figure 6. Inhibition of vesicular release caused unmyelinated segments via suppression of oligodendrogenesis at NL region.

(A) Timeline of experiments. *In ovo* electroporation and A3V transfection was performed at E2 (HH Stage 11–12) and at E2–3 (HH Stage 15–16), respectively, and brainstem was observed at E15 in (L–M) and at E21 in (C–K).

(B) A3V-eTeNT expressing GFP-tagged eTeNT was injected into neural tube to inhibit vesicular release from NM axons.

(C–I) eTeNT caused unmyelinated segments at NL region without affecting internodal length. Nodal distribution along a single NM axon at NL region for A3V-GFP (C) and A3V-eTeNT (D). Arrowheads indicate branch points of collaterals. Each number corresponds to high-magnification images of node. Each internode was labeled alternately with red and green lines, while unmyelinated segments were

labeled with yellow broken lines for A3V-eTeNT. “Unmyelinated segment” was identified as a non-overlapping axonal segment formed by a pair of heminodes, while internodes were classified into “internode w/o heminode” and “internode w/ heminode” according to the types of nodes at the ends (E) (see Methods). Percentages of heminode and mature/immature node (F), and internodal length (H) at tract and NL regions. Percentage of unmyelinated segments in the axon over 200um (G), and internodal length w/o and w/ heminode for A3V-eTeNT (I) at NL region. F: GFP (n=124) and eTeNT (n=160) at tract, GFP (n=186) and eTeNT (n=270) at NL. G: n=29 axons for GFP, n=38 axons for eTeNT. H: GFP (n=62) and eTeNT (n=80) at tract, GFP (n=178) and eTeNT (n=178) at NL. I: Internode w/o (n=115) and w/ (n=63) heminode.

(J–K) eTeNT did not affect oligodendrocyte morphology at NL region. Magnified images of single oligodendrocytes after A3V-eTeNT transfection (J). Number, total length, average length of myelins per oligodendrocyte were compared between A3V-eTeNT and A3V-GFP (Figure 4 H–J) and shown as a ratio (K; n=29).

(L–M) eTeNT suppressed of oligodendrogenesis at the NL region. Immunostainings of Nkx2.2 (OPC marker) and BrdU (proliferating cell marker) at E15 for A3V-GFP (upper) and A3V-eTeNT (lower) (L). Yellow and red arrowheads indicate tract and NL regions, respectively. Relative density of Nkx2.2- and BrdU-positive cells between tract and NL regions (M). A3V-eTeNT reduced the density of these cells specifically at NL region and abolished the difference between the regions. M: N=4 chicks for GFP, N=6 chicks for eTeNT.

Chi-square test (F), Wilcoxon rank sum test (G) and Two-tailed T-test (H, I, K, M): *$p < 0.05$, **$p < 0.01$, n.s., not significant.

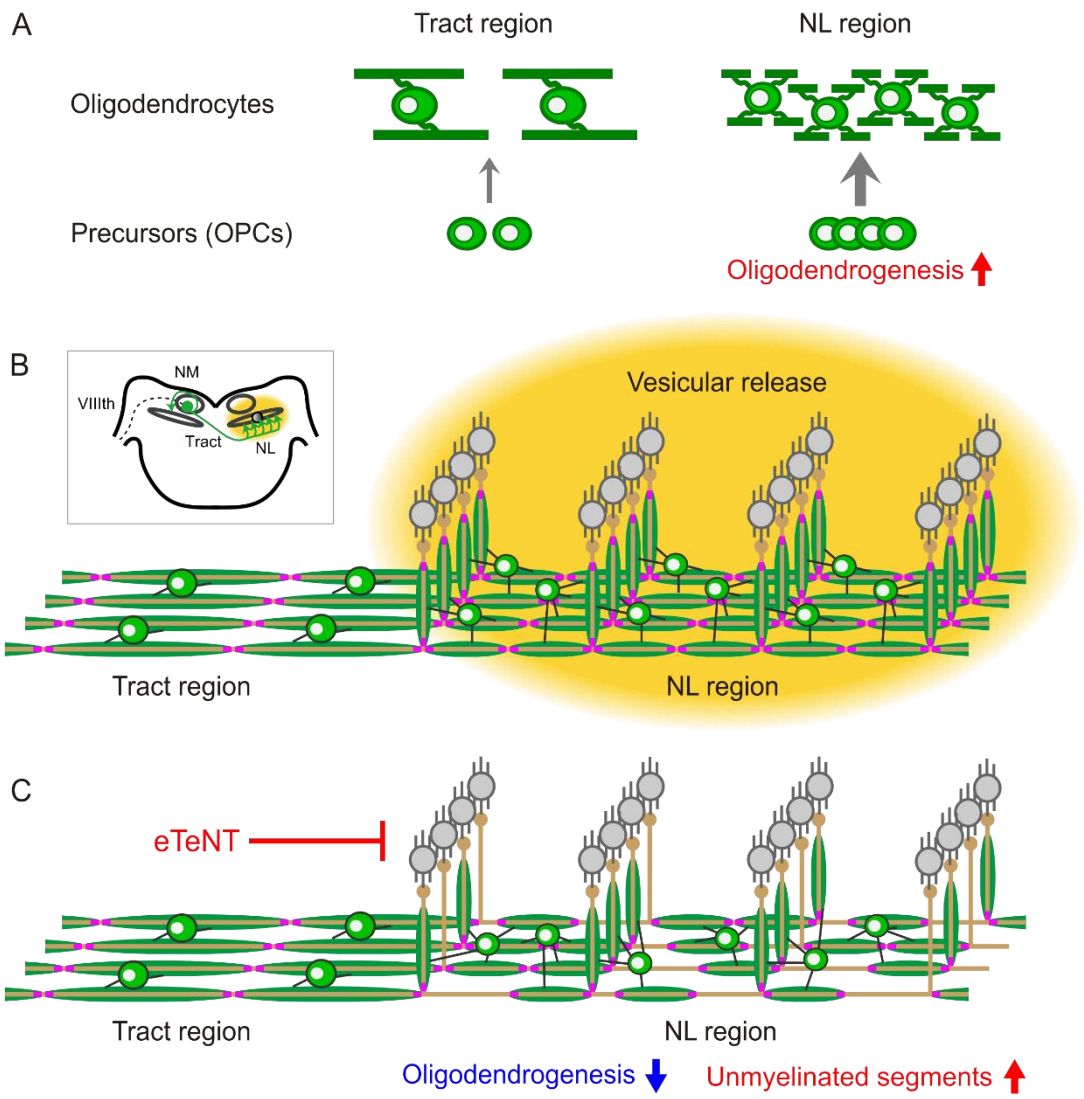

Figure 7. Regional heterogeneity of oligodendrocytes and adaptive oligodendrogenesis underlie the biased nodal distribution pattern along NM axons.

(A) Morphology of oligodendrocytes, such as the number and length of myelins, is determined intrinsically at each region; those at NL region have larger numbers of short myelins compared to those at tract region. In addition, adaptive oligodendrogenesis increases the density of oligodendrocytes specifically at NL region.

(B-C) Nodal distribution primarily reflects the length of myelins at each region (B). Inhibition of vesicular release from NM axons by eTeNT suppressed adaptive oligodendrogenesis and caused unmyelinated segments at NL region without altering oligodendrocyte morphology and internodal length (C), suggesting the importance of adaptive oligodendrogenesis in myelinating the entire axons with the short myelins at NL region. Thus, intrinsic and adaptive properties of oligodendrocytes play a pivotal role in shaping the region-specific nodal distribution along NM axons.

Video 1. High-resolution 3D serial images of oligodendrocytes at the tract region of a 200-µm-thick brainstem slice. NM axons were densely labeled with GFP (green) and mature oligodendrocytes (magenta) were sparsely labeled with paltdTomato. The three z-stack images were stitched together. Field of view: 614.89×222.08×180.9 µm.

Video 2. High-resolution 3D serial images of oligodendrocytes at the NL region of a 200-µm-thick brainstem slice. NM axons were densely labeled with GFP (green) and mature oligodendrocytes (magenta) were sparsely labeled with paltdTomato. The three z-stack images were stitched together. Field of view: 626.58×222.08×184.5 µm.

Video 3. High-resolution 3D serial images of nodal distribution along NM axon at the tract and NL regions of a 200-µm-thick brainstem slice.
NM axons were sparsely labeled with GFP (green) and Caspr (magenta) was immunostained. The six z-stack images were stitched together. Field of view: 1237.35×217.97×185.4 µm.